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Behaviour of α-Aminoadipylcysteine and Glutamylcysteine in the Presence of Intact and Disrupted Mycelium of a *Cephalosporium* sp.

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1. δ-(L-α-Aminoadipyl)-L-cysteine, the corresponding D- and DL-α-aminoadipyl isomers, δ-(DL-α-amino[6-^{14}C]adipyl)-L-cysteine and γ- and α-L-glutamyl-L-cysteine were synthesized. 2. The behaviour of δ-(L-aminoadipyl)-L-cysteine and the corresponding D- and DL-α-aminoadipyl isomers was studied in the presence of suspensions of intact mycelium of a *Cephalosporium* sp., suspensions treated ultrasonically and extracts obtained by grinding with alumina. 3. With intact mycelium the L-α-aminoadipyl isomer was removed more rapidly from the extracellular fluid than the corresponding D-isomer. 4. Addition of δ-(DL-α-amino[6-^{14}C]adipyl)-L-cysteine to suspensions of intact mycelium led to the labelling of extracellular and intracellular penicillin N and cephalosporin C, but also to extensive hydrolysis of the dipeptide. 5. Broken-cell systems hydrolysed δ-(L-α-aminoadipyl)-L-cysteine and the corresponding D-α-aminoadipyl isomer, but the former was hydrolysed more readily than the latter. 6. γ- and α-L-Glutamyl-L-cysteine were also hydrolysed but δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine was not. 7. Only part of the enzyme activity in broken-cell systems responsible for the hydrolysis of δ-(α-aminoadipyl)-L-cysteine was present in the supernatant obtained on centrifugation at 20000 *g*. 8. Possible implications of these findings are discussed.

In preliminary experiments designed to throw light on the possible role of δ-(α-aminoadipyl)-cysteine in the biosynthesis of δ-(α-aminoadipyl)-cysteinylvaline, penicillin N and cephalosporin C by a *Cephalosporium* sp., it appeared that a sample of the dipeptide prepared from DL-α-aminoadipic acid and L-cysteine was hydrolysed both by whole mycelium and by broken-cell systems. This hydrolysis was clearly relevant to the efficiency with which the dipeptide could be used intact in biosynthesis. It was also of interest because no enzyme has yet been reported that will effectively remove the δ-(D-α-aminoadipyl) side chain from cephalosporin C. In contrast, acylases are known which will remove phenylacetyl or other non-polar acyl groups not only from the corresponding penicillins but also from the *N*-acyl derivatives of a variety of amino acids (Cole, 1964; Kaufmann & Bauer, 1964).

The enzymic hydrolysis of different optical isomers of δ-(α-aminoadipyl)cysteine, which had been chemically synthesized, was consequently studied in more detail. Parallel studies were made with γ-L-glutamyl-L-cysteine as a model compound, since this dipeptide is known to be an intermediate in the biosynthesis of glutathione in yeast.

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EXPERIMENTAL

Paper chromatography and electrophoresis, radioautography and the counting of radioactive compounds on planchets and on paper were carried out as described by Smith, Warren, Newton & Abraham (1967). Electrometric titrations were carried out at 20° with a type TTT1a automatic titrator in conjunction with an SBR2c Titrigraph and a type TTA31 titration assembly from Radiometer, Copenhagen, Denmark. Determinations of protein were made by the method of Lowry, Rosebrough, Farr & Randall (1951) and of glutathione reductase activity as described by Beutler, Duron & Kelly (1963). Thiol groups were determined by the method of Ellman (1959) and detected on paper by spraying with starch–iodine (Sneath & Collins, 1961). Oxidation of thiol compounds to the corresponding sulphonic acids with performic acid was carried out as described by Smith *et al.* (1967). Tests for the presence of 6-oxopiperidine-2-carboxylic acid after electrophoresis on paper at pH 4·5 (Trown, Smith & Abraham, 1963) were made by the method of Rydon & Smith (1952).

DL-α-Amino[6-^{14}C]adipic acid (4·4 μc/μmole) was obtained from Calbiochem, Lucerne, Switzerland. The disulphide of δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine was kindly supplied by Professor Rudinger. *S*-Benzyl-L-cysteine was prepared as described by Hegedus (1948) and the corresponding ethyl ester hydrochloride as described by Harington & Pitt-Rivers (1944). Some of the reductions with Na in liquid NH_3 were carried out in the apparatus designed by Nesvadba & Roth (1967).

N-Benzyloxycarbonyl-δ-(α-aminoadipyl)hydrazide. The D-isomer (m.p. 128–130°) was prepared as described by Abraham & Newton (1954) except that at the final stage the solution was adjusted to pH 3·6 with 4M-HCl, when the product crystallized. The DL-isomer (m.p. 112–113°) and the L-isomer (m.p. 128–129°) were prepared in a similar manner.

N-Benzyloxycarbonyl-δ-(D-α-aminoadipyl)-S-benzyl-L-cysteine. The above hydrazide (310 mg.) was used to prepare a dry $CHCl_3$ solution of the azide (Abraham & Newton, 1954) and the latter was added to a solution of *S*-benzyl-L-cysteine ethyl ester [prepared from 750 mg. of ester hydrochloride by the method of Hillman (1946) for the preparation of glycine ethyl ester] in $CHCl_3$ at 0°. The subsequent stages were similar to those in the synthesis of δ-(benzyloxycarbonyl-α-aminoadipyl)glycine (Abraham & Newton, 1954). The product was obtained as an oil (270 mg.).

δ-(α-Aminoadipyl)-L-cysteine. The product from the preceding experiment (270 mg.) was dissolved in liquid NH_3 (15 ml.) in a flask fitted with a soda-lime tube. Na (100–150 mg.) was added until the solution was permanently blue and the colour was then discharged with NH_4Cl. After removal of the NH_3 the residue was dissolved in 0·2·5M-H_2SO_4 (6 ml.) and the solution extracted twice with ether. Ether was removed from the aqueous layer *in vacuo*, and the mercury salt of the dipeptide was precipitated by the addition of $HgSO_4$–H_2SO_4 reagent (du Vigneaud & Miller, 1952). The mercury salt was washed twice by centrifugation with boiled water, suspended in boiled water (5 ml.) and decomposed with H_2S. After removal of the HgS by centrifugation the supernatant was evacuated to remove H_2S. The solution was brought to pH 2 with 2·5M-H_2SO_4, warmed to 40° and a suspension of red Cu_2O (du Vigneaud & Miller, 1952) in water was added dropwise until no more precipitate appeared. The crystalline cuprous mercaptide was washed by centrifugation with boiled water until the washings were free of sulphate, then suspended in boiled water (3 ml.) and decomposed with H_2S. After removal of the precipitated sulphide by centrifugation, H_2S was removed *in vacuo* from the supernatant and the latter was freeze-dried (72 mg.). On exposure to air the product increased in weight by 12%, corresponding to nearly 2 H_2O/molecule. (Found: C, 29·8; H, 5·4; N, 7·6. $C_9H_{16}O_5N_2S,2H_2O$ requires C, 30·2; H, 5·6; N, 7·8%.)

The corresponding dipeptides from DL- and L-α-aminoadipic acid were prepared in a similar manner. All three compounds showed a thiol content which was about 97% of the theoretical value, and could be detected on paper by both ninhydrin and starch–iodine. On hydrolysis with 6M-HCl for 18 hr. at 105° they yielded α-aminoadipic acid and a mixture of cysteine and cystine.

Bis-δ-(DL-α-aminoadipyl)-L-cystine. Excess of Cu was removed with H_2S from the supernatant remaining after precipitation of the cuprous mercaptide (25 mg.). The resulting supernatant was brought to pH 2·8 with saturated $Ba(OH)_2$; the $BaSO_4$ was removed by filtration and the filtrate was freeze-dried (20 mg.).

This product was detectable on paper by coloration with ninhydrin, but gave no reaction with starch–iodine. Hydrolysis with 6M-HCl for 18 hr. at 105° followed by two-dimensional paper electrophoresis (pH 4·5) and chromatography (butan-1-ol–acetic acid–water, 4:1:4, by vol.) revealed only α-aminoadipic acid and cystine in approximately equal amounts.

δ-(DL-α-Amino-[6-^{14}C]-adipyl)-L-cysteine. This was prepared from a mixture of unlabelled DL-α-aminoadipic acid (56 mg.) and DL-α-amino[6-^{14}C]adipic acid (50 μc; 4·40 mc/m-mole). The procedure was as described under 'δ-(α-Aminoadipyl)-L-cysteine' as far as the decomposition of the mercury salt. The supernatant from the HgS was evacuated to remove H_2S, and saturated $Ba(OH)_2$ was added until no more $BaSO_4$ appeared. The $BaSO_4$ was removed by filtration and the filtrate was freeze-dried (3·3 mg.; 0·14 mc/m-mole). On paper electrophoresis at pH 4·5 and chromatography in butan-1-ol–acetic acid–water the product was indistinguishable from the corresponding unlabelled dipeptides when detected by ninhydrin, starch–iodine and radioautography.

L-Glutamyl-L-cysteine (mixture of α- and γ-isomers). *N*-Benzyloxycarbonyl-L-glutamylhydrazide (470 mg.) (Le Quesne & Young, 1950), was converted into the azide, coupled with *S*-benzyl-L-cysteine ethyl ester, and the ester group was removed by alkaline hydrolysis. Electrophoresis of the free acid on paper at pH 4·5, followed by treatment of the paper by the method of Rydon & Smith (1952), showed the presence of two compounds that had migrated slightly further towards the anode than had aspartic acid and glutamic acid respectively. The mixture was converted into a mixture of two L-glutamyl-L-cysteines (90 mg.) in the manner described for the preparation of δ-(α-aminoadipyl)-cysteine. On paper electrophoresis at pH 4·5, approx. 30% of the material migrated towards the anode 0·75 times as far as glutamic acid (α-isomer), and the remainder 1·4 times as far as glutamic acid (γ-isomer). Both compounds were detected by coloration with ninhydrin and reaction with starch–iodine.

The supernatant remaining after precipitation of the cuprous mercaptide yielded 72 mg. of material that was not distinguishable by paper electrophoresis from that obtained by decomposition of the cuprous mercaptide. There was no evidence of the presence of bis-(L-glutamyl)-L-cystine, since no spot was observed that gave a positive ninhydrin reaction but a negative starch–iodine reaction.

Separation of α- and γ-L-glutamyl-L-cysteine. The mixture of dipeptides (50 mg.) was dissolved in water (2 ml.) and applied to a column (10 cm. × 1 cm. diam.) of Dowex 1 (X8; acetate form; 200–400 mesh). Elution was carried out with 0·5M-acetic acid; fractions (2 ml.) were collected every 5 min. Analysis of the fractions (2 × 5 μl.) by electrophoresis on paper at pH 4·5, followed by coloration with ninhydrin, indicated that the α-isomer was present in fractions 7–9, and the γ-isomer in fractions 33–51.

Fractions 7–9 were combined and freeze-dried. The resulting α-L-glutamyl-L-cysteine (7 mg.) behaved as a single substance when subjected to electrophoresis at pH 4·5 and to electrophoresis at pH 1·8 after oxidation with performic acid.

The preparation of γ-L-glutamyl-L-cysteine from fractions 33–51 (38 mg.) behaved as a single substance when subjected to electrophoresis on paper at pH 4·5. On exposure to air the preparation increased in weight by 2·8%, corresponding to nearly 0·5 H_2O/molecule. (Found: C, 36·8; H, 6·0; N, 11·6. $C_8H_{14}O_5N_2S,\frac{1}{2}H_2O$ requires C, 37·0; H, 5·8; N, 10·8%.)

Both peptides were detected on paper by starch–iodine and by ninhydrin. After hydrolysis with 6M-HCl for 18 hr. at 105° only glutamic acid and cysteine (or cystine) were revealed by paper chromatography and electrophoresis.

Reduction of the disulphide of δ-(L-α-*aminoadipyl*)-L-*cysteinyl*-L-*valine*. This disulphide (23 mg.) was reduced with Na in liquid NH_3 (15 ml.) and the product was precipitated as a mercury salt as described for δ-(α-aminoadipyl)-L-cysteine. Saturated $Ba(OH)_2$ was added to the supernatant obtained after decomposition of the mercury salt until no more $BaSO_4$ appeared; the $BaSO_4$ was removed by filtration and the filtrate was freeze-dried (6 mg.). The amount of material was insufficient for further precipitation with red Cu_2O suspension (du Vigneaud & Miller, 1952). However, when the product was mixed with GSH (1:4 or 1:20, w/w) a crystalline cuprous mercaptide was obtained, which, after decomposition and analysis by electrophoresis on paper, was found to contain approximately the correct proportions of the two tripeptides. Measurement of the thiol content of the δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine showed that approx. 85% of the final product was in the reduced form.

Suspensions of washed mycelium. Cephalosporium sp., mutant C91 (Smith *et al.* 1967), was grown in a chemically defined medium in shake flasks and the mycelium was harvested 72 hr. after inoculation, washed with water and resuspended in water as described by Smith *et al.* (1967). Shake flasks (50 ml.) containing suspensions of washed mycelium (1 g. in 5 ml.) were shaken on a rotary shaker (200 rev./min. and 2 in. throw) at 27·5°.

Ultrasonic treatment of mycelium. Washed mycelium was resuspended in water and the suspension (1 g. of damp-dry mycelium/5 ml. of water) subjected to ultrasonic treatment for 30 min. as described by Smith *et al.* (1967). Part of the treated suspension was then centrifuged for 30 min. at 20000 *g* and the supernatant was removed. The latter contained about 3·7 mg. of protein/ml.

Disruption of mycelium with alumina. Alumina (200–400 mesh; Savory and Moore, London, W.1) was stirred with water, and dilute NaOH was added until the pH of the supernatant increased to 7·5. It was then filtered, washed with water and dried at 37°. Resuspension of this alumina in water gave a supernatant solution with pH 7·2–7·5. Damp-dry mycelium was ground by hand in a mortar with an equal weight of dry alumina for 15 min. at 5–10°. A buffered salt solution described by Demain (1963) containing 0·5 M-mannitol (1·2 ml./10 g. of damp-dry mycelium) was added and grinding was continued for a further 5 min. The paste was spun at 4500 *g* for 4 min. The turbid supernatant, which was free from whole and broken mycelial cells, was removed and used immediately. The supernatant contained about 35 mg. of protein/ml.

Incubation of peptides in broken-cell systems. The various dipeptides were dissolved in 1 equivalent of M-$NaHCO_3$ solution before being added to the whole ultrasonically-treated suspension, the 20000 *g* supernatant from it, or the extract obtained by grinding with alumina. All incubations were carried out at 27·5°. No energy-generating system was added.

Measurement of antibiotics, dipeptides and their constituent amino acids on paper. Radioactive penicillin N and cephalosporin C were measured by counting spots on paper after electrophoresis at pH 4·0 followed by chromatography in butan-1-ol–acetic acid–water (4:1:5, by vol.), as described by Smith *et al.* (1967). α-Amino[6-^{14}C]adipic acid was counted after electrophoresis of samples on paper (70 v/cm. for 30 min.) at pH 4·5.

In other cases approximate estimations of the amounts of peptides and their constituent amino acids in the systems used were made by visual comparison of spots on paper coloured by ninhydrin with those of known amounts of the appropriate compounds used as markers. α-Aminoadipic acid was measured after electrophoresis at pH 4·5, when it was well separated from other ninhydrin-positive substances.

In addition, the dipeptides, and cysteine derived from them, were determined after oxidation to the corresponding sulphonic acids. At the end of the incubation period the mixture (0·5–1·0 ml.) was centrifuged at 20000 *g* for 30 min. and the supernatant was freeze-dried. For further analysis the residues were desalted. Residues from the ultrasonic extract or supernatant were extracted into 70% (v/v) ethanol (2 × 200 μl.), and the ethanol extracts were diluted with water and freeze-dried. Half of the freeze-dried product was oxidized with performic acid as described by Smith *et al.* (1967). The residue from the extract obtained by grinding with alumina was dissolved in water (1 ml.), and the solution was added to a column (5 cm. × 1 cm. diam.) of Dowex 50 (X8; H^+ form; 200–400 mesh). The column was washed with water (6 ml.), and peptides and amino acids were eluted with M-pyridine. The eluate was freeze-dried and half of the residue (3–9 mg.) was oxidized with performic acid, as described by Smith *et al.* (1967). Samples corresponding to one-fifth or one-tenth of the product were subjected to electrophoresis on paper at pH 1·8 (70 v/cm. for 2 hr.). Cysteic acid, δ-(α-aminoadipyl)cysteic acid and γ- and α-glutamylcysteic acid were well separated from each other and from other substances in the mycelial extracts, which gave significant ninhydrin reactions under the conditions used. γ-Glutamylcysteic acid migrated to a position close to that of glutathionesulphonic acid, but the amount of the latter in the samples tested was at least an order of magnitude lower than the initial amount of the former.

RESULTS

Synthesis and some properties of α-aminoadipylcysteine and glutamylcysteine

δ-(L-α-Aminoadipyl)-L-cysteine, the corresponding D- and DL-α-aminoadipyl isomers and δ-(DL-α-amino[6-^{14}C]adipyl)-L-cysteine were synthesized by the azide-coupling route from the δ-(benzyloxycarbonyl-α-aminoadipyl)hydrazides and *S*-benzyl-L-cysteine ethyl ester. The peptides yielded crystalline cuprous mercaptides. A similar procedure, starting from the benzyloxycarbonyl-L-glutamyl-γ-hydrazide, led to a mixture of γ- and α-L-glutamyl-L-cysteine, which were separated by chromatography on Dowex 1 (X8; acetate form).

Electrometric titration of δ-(α-aminoadipyl)-cysteine revealed the presence of four ionizable groups with pK_a values of 10·6 (thiol), 9·6 (ammonium), 3·5 (*C*-terminal carboxyl) and < 3·0 (*N*-terminal carboxyl) respectively. γ-Glutamylcysteine showed corresponding pK_a values of 10·4, 9·1, 3·1 and < 3·0. The corresponding pK_a values for α-glutamylcysteine were 9·3, 7·6, 3·1 and 3·7 respectively.

The relative mobilities of the different dipeptides and of glutathione on paper at pH 4·5 and pH 1·8,

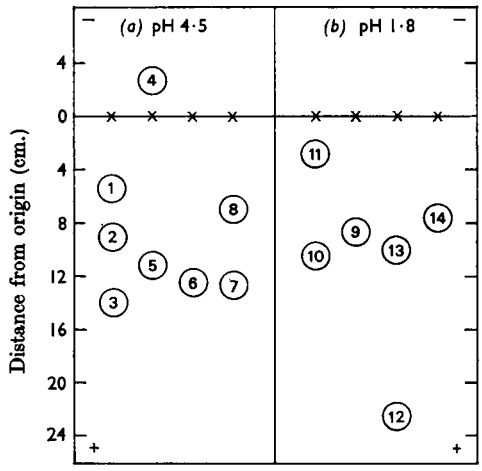

Fig. 1. Electrophoresis of peptides and amino acids on paper (70v/cm.): (*a*) in pyridine–acetate at pH4·5 for 30min., and (*b*) in acetic acid–formic acid at pH1·8 for 2hr. after oxidation of samples with performic acid. (*a*): 1, α-Aminoadipic acid; 2, glutamic acid; 3, aspartic acid; 4, cysteine; 5, δ-(α-aminoadipyl)cysteine; 6, bis-δ-(α-aminoadipyl)-cystine; 7, γ-glutamylcysteine; 8, α-glutamylcysteine. (*b*): 9, δ-(α-aminoadipyl)cysteic acid; 10, γ-glutamylcysteic acid; 11, α-glutamylcysteic acid; 12, cysteic acid; 13, glutathionesulphonic acid; 14, δ-(α-aminoadipyl)cysteicyl-valine.

before and after oxidation to the corresponding sulphonic acids, are given in Fig. 1. The mobilities of δ-(α-aminoadipyl)cysteicylvaline and of certain amino acids under the same conditions are given for comparison.

Behaviour of δ-(α-aminoadipyl)-L-cysteine in the presence of aerated washed mycelium

Neutralized solutions of δ-linked DL-, D- and L-(α-aminoadipyl)-L-cysteine and of L-cysteine were added to suspensions in water of washed mycelium in shake flasks at 27·5° to give a final concentration of the added compound of 0·7μmole/ml. Fig. 2 shows the disappearance of thiol groups from the extracellular fluid, expressed as a percentage of the corresponding value for the control flasks, in which the neutralized compounds were shaken with boiled water alone. The peptide containing the L-isomer of α-aminoadipic acid disappeared more rapidly than that containing the D-isomer. After 6hr. the values for the control flasks had fallen to approx. 70% of the original values.

Fig. 3 shows the rate of disappearance of radioactivity from the extracellular fluid when a solution of δ-(DL-α-amino[6-^{14}C]adipyl)-L-cysteine sodium salt was added to a suspension of washed mycelium

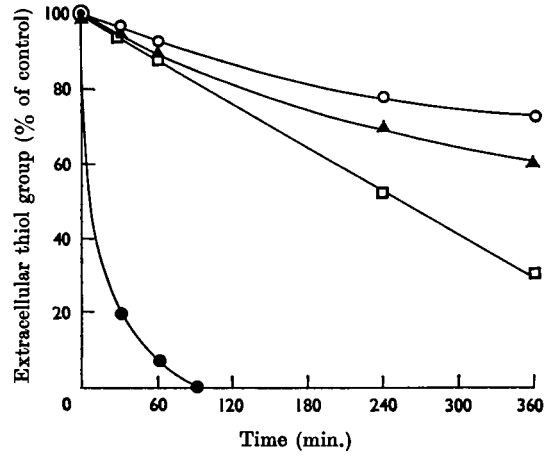

Fig. 2. Removal of δ-linked D-, DL- and L-(α-aminoadipyl)-L-cysteine and of L-cysteine from the extracellular fluid by suspensions of washed mycelium, as estimated by the decrease in number of thiol groups. The initial concentration of each substance was 0·7μmole/ml. ○, δ-(D-α-Aminoadipyl)-L-cysteine; ▲, δ-(DL-α-aminoadipyl)-L-cysteine; □, δ-(L-α-aminoadipyl)-L-cysteine; ●, L-cysteine.

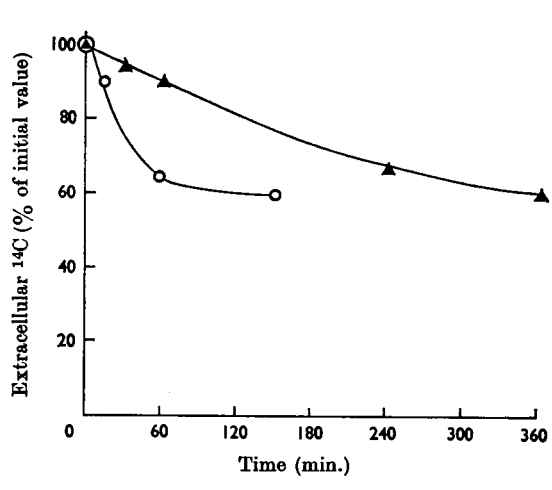

Fig. 3. Removal of δ-(DL-α-amino[6-^{14}C]adipyl)-L-cysteine and DL-α-amino[6-^{14}C]adipic acid from the extracellular fluid by suspensions of washed mycelium, as estimated by the decrease in radioactivity. The initial concentrations were 0·45μmole/ml. and 0·3μmole/ml. respectively. ▲, δ-(DL-α-Amino[6-^{14}C]adipyl)-L-cysteine; ○, DL-α-amino-[6-^{14}C]adipic acid.

(to give a final concentration of 0·45μmole/ml. and 0·062μc/ml.) in shake flasks at 27·5°. After incubation for 6hr. the extracellular fluid was removed by filtration and freeze-dried. The mycelial pellet was washed well with water, then

Table 1. *Uptake and distribution of* ^{14}C *from* δ-(DL-α-*amino*-[6-^{14}C]*adipyl*)-L-*cysteine with suspensions of washed mycelium*

The labelled dipeptide (final concn. 0·2 mg./ml., 0·7 μmole/ml.) was incubated for 6 hr. at 27·5° in shake flasks with suspensions of mycelium in water (5 ml.). The radioactivities of samples (one-tenth of the total) of the whole intracellular pool and extracellular fluid were determined by counting on planchets. The radioactivities of individual substances were determined by counting on paper (*a*) after electrophoresis at pH 4·0 and chromatography or (*b*) after performic acid oxidation and electrophoresis at pH 1·8 (for details see the text). Abbreviations: AC, δ-(DL-α-amino-[6-^{14}C]adipyl)-L-cysteine; Ceph C, cephalosporin C; Pen N, penicillin N.

	Radioactivity (nc)					
		AC		α-Amino-adipic acid	Ceph C	Pen N
Mycelial fraction ...	Total	(*a*)	(*b*)	(*a*)	(*a*)	(*a*)
Intracellular pool	68	—	2·6	62	3·6	3·1
Extracellular fluid	83	20	21	47	6·4	7·3

shaken for 5 min. with 2·5 ml. of ice-cold 70% (v/v) ethanol and for 1 min. with 2·5 ml. of ice-cold water to extract amino acids and other substances from the intracellular pool. These ethanol and water extracts were combined and freeze-dried.

The extracellular material and the mycelial extract were analysed by electrophoresis and chromatography on paper before and after performic acid oxidation. The amounts of radioactivity in the spots on paper located by radioautography are given in Table 1.

In the presence of the intact mycelium labelled α-aminoadipic acid from δ-(α-amino[^{14}C]adipyl)-cysteine was used as a precursor of the side chain of cephalosporin C and penicillin N. More free labelled α-aminoadipic acid was found in the intracellular pool than in the extracellular fluid, and only a very small amount of dipeptide was detected in the intracellular fluid.

Behaviour of δ-(α-aminoadipyl)-L-cysteine and γ- and α-glutamylcysteine in the presence of broken-cell systems

Experiments with mycelium broken by ultrasonic treatment. Neutralized solutions of δ-linked D-, L- and DL-(α-aminoadipyl)-L-cysteine respectively were added separately to suspensions of mycelium that had been treated ultrasonically, and to the 20000 *g* supernatants from these suspensions, to give a final peptide concentration of 2 mg./ml. (7 μmoles/ml.). The mixtures were incubated at 27·5° for 5 hr. Samples were taken before incubation and at 1, 3 and 5 hr., centrifuged at 3000 *g* for 5 min. and the supernatants (20 μl.) were analysed by electrophoresis on paper at pH 4·5 followed by coloration with ninhydrin. Semi-quantitative estimates of the amounts of α-aminoadipic acid present are given in Table 2. These showed that in each case more α-aminoadipic acid was produced when the peptide was incubated with the whole suspension than with the supernatant alone. Incubation of the peptides with a suspension previously heated at 100° for 30 min. produced no α-aminoadipic acid. The peptide synthesized from the L-isomer of α-aminoadipic acid was hydrolysed more extensively than that from the D-isomer. Determinations, after oxidation with performic acid, of cysteic acid and dipeptide in the sulphonic acid form confirmed that hydrolysis occurred more readily with the whole suspension than with the corresponding supernatant. They also confirmed that the L-α-aminoadipyl peptide was more readily catabolized than the D-α-aminoadipyl isomer (Table 2).

Further experiments were done to determine the relative efficiency with which the whole ultrasonically treated mycelium, and the supernatant from it, catalysed the formation of α-aminoadipic acid from the dipeptide. DL-(α-Amino[6-^{14}C]adipyl)-L-cysteine sodium salt was added to samples of the whole suspension and the supernatant to give a final concentration of 2·00 μmoles/ml. and 0·28 μc/ml. in each case. Samples were taken before incubation, and at 2 and 4 hr., for analysis by electrophoresis on paper at pH 4·5, followed by radioautography. α-Aminoadipic acid was formed more rapidly when the dipeptide was incubated with the whole suspension than with the supernatant, and the dipeptide disappeared at a correspondingly more rapid rate (Table 3). There was no indication that any of the dipeptide was converted into 6-oxopiperidine-2-carboxylic acid.

Addition of γ-L-glutamyl-L-cysteine to the whole suspension and to the 20000 *g* supernatant, followed by sampling and analysis as described above, showed that this dipeptide also disappeared at a more rapid rate when incubated with the whole ultrasonic extract, and a correspondingly larger amount of free cysteic acid was detected after oxidation (Table 2).

Experiments with extracts of mycelium ground with alumina. Neutralized solutions of δ-(L-α-aminoadipyl)-L-cysteine, γ-L-glutamyl-L-cysteine and α-L-glutamyl-L-cysteine were added to the alumina extract to give a final concentration of 7 μmoles/ml. The mixtures were incubated at 27·5° for 5 hr. Immediately before incubation and at 1, 3 and 5 hr. samples were taken for analysis by electrophoresis on paper, followed by coloration with ninhydrin. It was clear that α-L-glutamyl-L-cysteine was broken down very rapidly, none

Table 2. *Hydrolysis of δ-(α-aminoadipyl)-L-cysteine and L-glutamyl-L-cysteine by fractions of disrupted mycelium*

Each dipeptide (final concn. 7 μmoles/ml.) was incubated for 5 hr. at 27·5° with 0·5 ml. of the preparations from disrupted mycelium (for details see the text). Samples (20 μl.) were analysed by electrophoresis on paper at pH 4·5 or (after oxidation with performic acid) at pH 1·8 and the spots were coloured with ninhydrin. Semi-quantitative estimations of the amounts of material in different spots were made visually, to within ±10 nmoles with dipeptides and ±5 nmoles with the amino acids. Abbreviations: AC, δ-(α-aminoadipyl)-L-cysteine; GC, L-glutamyl-L-cysteine.

		Amount (nmoles) of								
		Dipeptide in			α-Aminoadipic acid in			Cysteic acid in		
Starting substance	Time (hr.)	Whole ultrasonic extract	Supernatant	Alumina extract	Whole ultrasonic extract	Supernatant	Alumina extract	Whole ultrasonic extract	Supernatant	Alumina extract
DL-AC	0	140	140		<5	<5		<5	<5	
	1	140	140		10	<10				
	3	120	120		30	10				
	5	100	120		40	20		40	20	
D-AC	0	140	140		<5	<5		<5	<5	
	1	140	140		<10	<10				
	3	120	140		10	10				
	5	120	140		20	10		20	10	
L-AC	0	140	140	140	<5	<5	<5	<5	<5	<5
	1	120	140	120	10	<10	20			
	3	100	120	90	30	10	60			
	5	70	100	50	60	30	90	60	30	60
γ-L-GC	0	140	140	140				<5	<5	<5
	1	120	120	100						
	3	90	100	70						
	5	50	70	40				90	60	100
α-L-GC	0			140						<5
	1			70						
	3			30						
	5			<10						120

remaining after 5 hr. γ-L-Glutamyl-L-cysteine was broken down less rapidly, but more so than δ-(L-α-aminoadipyl)-L-cysteine (Table 2).

Effect of broken-cell systems on δ-(α-aminoadipyl)-cyst(e)inylvaline. Neutralized solutions of both δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine and its disulphide were added separately (to give final concentrations of 2 mg./ml. and 4 mg./ml. respectively) to portions of the whole mycelium after ultrasonic treatment and to the 20000 *g* supernatant. The mixtures were incubated at 27·5° for 5 hr. Samples (20 μl.) were taken before incubation and at 1, 3 and 5 hr. for analysis by electrophoresis on paper at pH 4·5, followed by coloration with ninhydrin. In neither case was the appearance of any α-aminoadipic acid noted.

The 20000 *g* supernatant had a glutathione reductase activity (NADPH-dependent) of 0·44 μmole of substrate/min./ml. at 25°; however, no reductase could be demonstrated with the disulphide of δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine as substrate.

DISCUSSION

The finding that only δ-(α-aminoadipyl)cysteine was obtained by synthesis from the δ-azide of *N*-benzyloxycarbonyl-α-aminoadipic acid, whereas a mixture of γ- and α-glutamylcysteine was obtained from the γ-glutamylazide, may be due to the inability of the α-aminoadipyl derivative to form an anhydride or cyclic pseudo-anhydride intermediate. The formation of such an anhydride from *N*-benzyloxycarbonylglutamyl-γ-azide was suggested by Sachs & Brand (1954). As expected, α-glutamylcysteine differed from the γ-glutamyl and δ-α-aminoadipyl peptides in the lower pK_a of the amino groups and the higher pK_a of the carboxyl group of its *N*-terminal residue (Abraham & Newton, 1954). The pK_a values assigned to the thiol groups of all three peptides are higher than that (8·7) assigned to the thiol group in glutathione (Calvin, 1954). This may be ascribed to the effect of the negative charge of the carboxylate ion of the *C*-terminal cysteine residues.

Table 3. *Addition of δ-(DL-α-amino[6-^{14}C]adipyl)-cysteine to mycelium subjected to ultrasonic treatment*

The labelled dipeptide (final concn. 2 μmoles/ml.) was incubated for 4 hr. at 27·5° with 0·5 ml. of the preparations from ultrasonically disrupted mycelium (for details see the text). Radioactivities of samples (20 μl.) were determined as in Table 1. Values in parentheses were obtained on samples of the final freeze-dried material representing one-tenth of the total. Abbreviation: AC, δ-(DL-α-amino[6-^{14}C]-adipyl)cysteine.

	Radioactivity (nc)				
		Found on paper with			
		Mycelium subjected to ultrasonic treatment (A) in		Supernatant from (A) in	
Time (min.)	Total applied to paper	AC	α-Amino-adipic acid	AC	α-Amino-adipic acid
5	5·6	5·6	0·3	5·6	0·05
120	5·6	4·5	1·1	5·6	0·3
240	5·6	4·0	1·1	5·3	0·5
240	(25)	(21)	(5·7)	(22)	(3·0)

In the first hour of incubation with intact aerated mycelium the rate of disappearance from the extracellular fluid of the ^{14}C from δ-(DL-α-amino-[^{14}C]adipyl)-L-cysteine (initial concn. 0·7 μmole/ml.) was only about one-fifth of that from DL-α-amino-[^{14}C]adipic acid in a comparable experiment (Fig. 3). The latter was due mainly to the rate of uptake of the L-isomer of α-aminoadipic acid, which was approximately proportional to its concentration until this approached 0·7 μmole/ml., with a rate constant for a first-order reaction which was about ten times that with the D-isomer (Warren, Newton & Abraham, 1967). Approximate estimations of the decrease in the concentration of extracellular dipeptide from the fall in the number of thiol groups, compared with that in a control solution in the absence of mycelium, showed that the apparent rate of disappearance of δ-(L-α-aminoadipyl)-L-cysteine was much greater than that of the corresponding D-α-aminoadipyl compound, and nearly twice that of the corresponding DL-α-aminoadipyl compound (Fig. 2). L-Cysteine was taken up much more rapidly than any of the peptides. Thus, if the dipeptides were able to enter the mycelium intact, apparently only the L-α-aminoadipyl isomer did so at an appreciable rate and this rate was considerably less than the rate of uptake of its constituent amino acids.

Other results, however, showed that the peptides did not remain intact. After 6 hr. 85% of the added DL-α-aminoadipyl peptide had been metabolized, and 72% of its α-aminoadipic acid residue was present as free α-aminoadipic acid (Table 1). Thus, extensive hydrolytic fission of both the L- and the D-α-aminoadipyl peptides had occurred, and both L- and some D-α-amino[^{14}C]adipic acid were presumably present in the intracellular pool. In experiments with disrupted mycelium both the L- and D-α-aminoadipyl peptides were hydrolysed, but the former to a greater extent than the latter.

Table 1 shows that 31% of the total ^{14}C added was present in extracellular α-amino[^{14}C]adipic acid 6 hr. after addition of the peptide to intact mycelium. This indicates that considerable hydrolysis took place near the surface of the mycelial cell before the peptide had entered the intracellular pool, since earlier experiments (Warren *et al.* 1967) showed that the pool of α-aminoadipic acid in the *Cephalosporium* sp. was expandable and that there was very little leakage of this amino acid into the extracellular fluid. The finding that less than 1% of the added labelled dipeptide was present as such in the intracellular pool was consistent with (though it did not establish) the hypothesis that most of the uptake of ^{14}C was preceded by hydrolysis of the peptide to α-aminoadipic acid and L-cysteine. The finding that the whole ultrasonically treated mycelium was more effective than the particle-free supernatant in catalysing hydrolysis of the dipeptides, which indicated that some of the enzyme was still particle-bound, was also consistent with the hypothesis that the enzyme was located in a cell boundary structure.

On the assumption that the presence of labelled α-aminoadipic acid in the intracellular pool was a consequence of the reactions:

$$\underset{(a)}{\text{α-Aminoadipylcysteine}} \xrightarrow{k_1} \underset{(x)}{\text{Extracellular α-aminoadipic acid}} \xrightarrow{k_2} \underset{(b)}{\text{Intracellular α-aminoadipic acid}}$$

and that k_1 and k_2 are rate constants for first-order reactions, then the rate of entry of α-aminoadipic acid would reach a maximum when [x] is a maximum, i.e. when

$$k_1[a-x] = k_2[x]$$

or

$$[x]/[a] = k_1/(k_1+k_2)$$

Thus, if $k_2 = 4k_1$ with L-α-aminoadipic acid, $[x]/[a] = 1/5$. The maximum extracellular concentration reached by the L-α-aminoadipic acid formed by hydrolysis of the dipeptide would then be one-fifth of the initial concentration of the

latter. The maximum rate of disappearance of ^{14}C in labelled dipeptide from the extracellular fluid would be one-fifth of that of the initial rate with the same molar concentration of labelled free α-aminoadipic acid. This approximated to the result shown in Fig. 2.

A major part, at least, of the ^{14}C found in penicillin N and cephalosporin C after addition of δ-(DL-α-amino[^{14}C]adipyl)-L-cysteine to the mycelial suspension could be incorporated via free α-amino-[^{14}C]-adipic acid as an intermediate. Simple hydrolysis appeared to represent the main metabolic pathway for the dipeptide since no evidence was found of transpeptidation or of the formation of 6-oxopiperidine-2-carboxylic acid.

Further experiments are thus required to establish the role of δ-linked L- or D-(α-amino-adipyl)cysteine, or both, in the biosynthesis of cephalosporin and penicillin.

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REFERENCES

Abraham, E. P. & Newton, G. G. F. (1954). *Biochem. J.* **58**, 266.

Beutler, E., Duron, O. & Kelly, B. M. (1963). *J. Lab. clin. Med.* **61**, 882.

Calvin, M. (1954). In *Glutathione: A Symposium*, p. 9. Ed. by Colowick, S. P., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H. New York: Academic Press Inc.

Cole, M. (1964). *Nature, Lond.*, **203**, 519.

Demain, A. L. (1963). *Clin. Med.* **70**, 2045.

du Vigneaud, V. & Miller, G. L. (1952). *Biochem. Prep.* **2**, 87.

Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.

Harington, C. R. & Pitt-Rivers, R. V. (1944). *Biochem. J.* **38**, 417.

Hegedus, B. (1948). *Helv. chim. Acta*, **31**, 737.

Hillman, G. von (1946). *Z. Naturf.* **1**, 682.

Kaufmann, W. & Bauer, K. (1964). *Nature, Lond.*, **203**, 520.

Le Quesne, W. J. & Young, G. T. (1950). *J. chem. Soc.* p. 1959.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Nesvadba, H. & Roth, H. (1967). *Mh. Chem.* **98**, 1432.

Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.

Sachs, H. & Brand, E. (1954). *J. Amer. chem. Soc.* **76**, 1815.

Smith, B., Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967). *Biochem. J.* **103**, 877.

Sneath, P. H. A. & Collins, J. F. (1961). *Biochem. J.* **79**, 512.

Trown, P. W., Smith, B. & Abraham, E. P. (1963). *Biochem. J.* **86**, 284.

Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967). *Biochem. J.* **103**, 891.